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Relationship between Virulence of *Mycobacterium avium* Strains and Induction of Tumor Necrosis Factor Alpha Production in Infected Mice and in In Vitro-Cultured Mouse Macrophages

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We studied the ability of two *Mycobacterium avium* strains with different virulences to induce tumor necrosis factor alpha (TNF) synthesis by mouse resident peritoneal macrophages (RPMϕ) in vitro in an experiment to look for a possible correlation between virulence and this TNF-inducing capacity. The low-virulence strain, 1983, induced significantly higher production of TNF by RPMϕ than did the high-virulence strain, ATCC 25291. TNF neutralization during culture of infected RPMϕ resulted in enhancement of growth of strain 1983 and had no effect on growth of strain ATCC 25291; TNF treatment of strain ATCC 25291-infected macrophages had no effect on mycobacterial growth. The extent of *M. avium* growth and the amount of TNF synthesis were independent of the presence of contaminating T cells or NK cells in the macrophage monolayers. Intraperitoneal administration of anti-TNF monoclonal antibodies to BALB/c mice infected intravenously with *M. avium* 1983 abrogated the elimination of the bacteria in the liver and caused a slight increase in bacterial growth in the spleen. Neutralization of TNF led to a minor increase in the proliferation of *M. avium* ATCC 25291 in the liver and spleen of BALB/c mice late in infection. Anti-TNF treatment did not affect the growth of the two *M. avium* strains in BALB/c.Bcg^r (C.D2) mice, suggesting that restriction of *M. avium* growth in these mycobacterium-resistant mice is TNF independent. In conclusion, the capacity of certain avirulent *M. avium* strains to induce TNF production by macrophages may limit their ability to proliferate both in vitro and in vivo.

Mycobacterium avium is a facultative intracellular pathogen of emerging importance in human disease, causing opportunistic infection in elderly individuals and in AIDS patients (29). These infections pose difficult problems for their management because of the resistance of *M. avium* to conventional antibiotics and antituberculous drugs.

Tumor necrosis factor alpha (TNF) is a cytokine that is produced mainly by macrophages/monocytes but is also secreted by other cells including B cells, T cells, NK cells, glial cells, and adipocytes (35). It affects the function of many cell types and is a mediator of inflammation and cellular immune responses (9, 22, 26, 34, 35).

TNF has been shown to play an important role in regulating the microbicidal activity of macrophages against several microorganisms (24, 32, 33), including *M. avium* (7, 13) and other mycobacteria (12, 22). In fact, TNF was reported to be involved in growth restriction of *M. avium*, both in vitro (7, 14, 17) and in vivo, during T-cell-independent and -dependent phases of *M. avium* infection (1, 3, 13). Induction of TNF production by mycobacteria has also been described (4, 11, 15, 17, 31). In fact, *M. leprae* induced TNF production in peripheral blood mononuclear cells from leprosy patients (4), and lipoarabinomannan from *M. tuberculosis* induced TNF secretion by mouse casein-elicited peritoneal and bone marrow-derived macrophages (BMMϕ) (11). It was also reported that different strains of *M. avium* stimulated TNF production by mouse BMMϕ (17) and human macrophages (15).

Different *M. avium* strains, living in different environments, may differ in virulence for mice (28). Furney et al. (17), working with two sets of *M. avium* strains, some able to proliferate in in vitro-cultured macrophages and others unable to do so, showed that the nonvirulent strains induced earlier TNF production by BMMϕ in vitro than did virulent strains. They also found that TNF addition to the infected macrophage cultures restricted the growth of the virulent strains of *M. avium*. These authors concluded that the capacity to induce early TNF production by macrophages may be relevant for determining the virulence of *M. avium* strains.

Another level of mouse innate resistance to mycobacteria depends on the expression of the *Bcg*/*Ity*/*Lsh* gene. This gene controls natural resistance or susceptibility of mice to infection by *M. bovis* BCG (19), *Salmonella typhimurium* (30), and *Leishmania donovani* (10). It also seems to play a role in resistance to *M. avium* infection (18), although evidence exists that other genes may also be involved (27). The *Bcg* gene is expressed by macrophages (20, 25). Earlier studies with *L. donovani* showed a differential expression of TNF by *Lsh*^{s}- and *Lsh*^{r}-primed BMMϕ; the authors found a correlation between TNF production and leishmanicidal activity (8).

In previous work, we investigated whether the ability to trigger TNF secretion could account for the difference in virulence observed between two isogenic morphotype variants of *M. avium* 2-151 (smooth-transparent [SmTr] and smooth-opaque [SmOp]) (3). Although we could not show a relationship between virulence of these morphotypes and capacity to induce TNF secretion, we found that one avirulent *M. avium* strain (strain 1983) was able to induce high TNF secretion in vitro (whereas strain 2-151 SmOp and SmTr were not), and so avirulence of this strain could be related to its capacity to trigger the secretion of TNF. To test this hypothesis further, we used two *M. avium* strains differing in virulence for mice, strain ATCC 25291 (high virulence) and strain 1983 (low virulence), both with an SmTr morphotype, to infect mouse resident peritoneal macrophages (RPMϕ) from mycobacterium-susceptible

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BALB/c mice. We studied TNF production in vitro by infected macrophages and the effects of recombinant TNF or anti-TNF antibody addition to RPMϕ on *M. avium* intracellular growth. To find out about the existence of a relationship between natural resistance and TNF production in the in vivo infection, we studied the effect of anti-TNF treatment on the in vivo growth of *M. avium* ATCC 25291 or 1983 in mice naturally resistant (*Bcg*^{r}) or susceptible (*Bcg*^{s}) to *M. avium* infections.

MATERIALS AND METHODS

Mice. Female BALB/c mice, 8 to 12 weeks old, were purchased from the Instituto Gulbenkian de Ciência, Oeiras, Portugal. BALB/c *Bcg*^{r} (C.D2) mice were bred at our facilities from parent mice generously donated by E. Skamene. Animals were kept under standard hygiene conditions, fed commercial chow, and given acidified drinking water ad libitum.

Reagents. Tween 80, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and saponin were purchased from Sigma, St. Louis, Mo. Middlebrook 7H9 and 7H10 media were obtained from Difco, Detroit, Mich. Hanks balanced salt solution, Dulbecco's modified Eagle's medium, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, and heat-inactivated fetal calf serum were purchased from Gibco, Paisley, United Kingdom. Recombinant mouse TNF was obtained from Genzyme, Cambridge, Mass. Lyophilized rabbit complement was purchased from Serotec, Oxford, England. All other reagents were purchased from Merck, Darmstadt, Germany.

Hybridomas and antibodies. The following hybridomas were used: 2.43, anti-Lyt 2.2 (ATCC TIB 210); GK 1.5, anti-L3T4 (ATCC TIB 207); PK 136, anti-NK 1.1; and MP6-XT22, monoclonal rat anti-mouse TNF (kindly donated by Paulo Vieira, DNAX Research Institute, Palo Alto, Calif.). All hybridomas were cultured until reaching exponential growth in Dulbecco's modified Eagle's medium containing 10 mM HEPES buffer and 10% fetal calf serum, and supernatants of 2.43, GK 1.5, and PK 136 were collected after culture centrifugation at 100 × *g* for 10 min. Anti-TNF was obtained from the ascites fluid of HSD nude mice, inoculated with the hybridoma MP6-XT22 from exponential growth culture, by protein G-agarose affinity chromatography. GL 113 anti-β-galactosidase immunoglobulin G1 was kindly donated by P. Vieira and used as an isotype control. Fluorescein isothiocyanate-conjugated rat anti-mouse CD4 or CD8 and phycoerythrin-conjugated hamster anti-mouse CD3-ε monoclonal antibodies were purchased from Pharmingen, San Diego, Calif.

Mycobacteria. *M. avium* ATCC 25291 (high virulence for mice [28]) and 1983 (low virulence for mice [28]), both of the SmTr colony morphotype, were grown in Middlebrook 7H9 medium containing 0.04% Tween 80 at 37°C until mid-log phase. The bacteria were harvested by centrifugation and resuspended in a small volume of saline containing 0.04% Tween 80. The suspension was briefly sonicated (15 s at 50 W) with a Branson sonifier to disrupt bacterial clumps, diluted, and frozen in aliquots at −70°C until use. The bacterial aliquots were thawed at 37°C and diluted to the desired concentration before being used for inoculation.

Macrophage culture. The peritoneal cells were collected from untreated BALB/c mice by washing the peritoneal cavity with Hanks balanced salt solution. The cells were centrifuged at 100 × *g* for 10 min, counted, and suspended at 3 × 10^6 cells per ml in Dulbecco's modified Eagle's medium containing 10 mM HEPES buffer and 10% fetal calf serum. A 1-ml volume of the cell suspension was placed in each well of 24-well tissue culture plates, and the plates were incubated for 2 h at 37°C in a humidified atmosphere containing 7% CO_2. Nonadherent cells were removed by washing with warm Hanks balanced salt solution, and the adherent cells were further incubated in Dulbecco's modified Eagle's medium containing HEPES buffer and fetal calf serum.

In one experiment, the peritoneal exudate cells were incubated with culture supernatants of the hybridomas GK 1.5, 2.43, and PK 136 in the presence of complement for 45 min and then washed twice before being used for adhesion.

In vitro infection. After removal of the nonadherent cells, the macrophage-enriched monolayers were inoculated with 10^7 CFU of *M. avium* ATCC 25291 or 1983 per well; the plates were centrifuged at 100 × *g* for 10 min and incubated for 2 h under the conditions described for macrophage adherence. For some wells, the supernatant was collected and TNF concentrations were immediately quantitated by the L929 bioassay. The extracellular bacteria were washed out, and the contents of a group of wells were then lysed with 0.1% saponin. Serial dilutions were seeded onto Middlebrook 7H10 medium with OADC supplement for CFU counts (time zero). The infected cells were maintained in culture for 7 days, and the same assays were performed after 3 and/or 7 days of infection. In some experiments, 5,000 neutralizing units of MP6-XT22 rabbit anti-mouse TNF or the same protein content of isotype control was added to the culture wells after removal of the nonphagocytosed bacteria. In other experiments, 500 U of recombinant mouse TNF per ml was added to the macrophage cultures each day.

Bioassay for TNF determination. TNF activity in culture supernatants from uninfected and infected RPMϕ was determined by an L929 biological cytotoxicity assay (16). Briefly, 0.1 ml of L929 cell suspension (3 × 10^5 cells per ml) was seeded per well of 96-well culture plates. After overnight incubation, 100-μl portions of serial dilutions from culture supernatants from uninfected, strain 1983-infected, and strain ATCC 25291-infected RPMϕ were added to the plates in the presence of 1 μg of actinomycin D per ml. Cell viability was measured 24 h later by incubating the monolayers with MTT. One unit of TNF corresponds to the amount of TNF required to produce a 50% reduction in the amount of formazan produced.

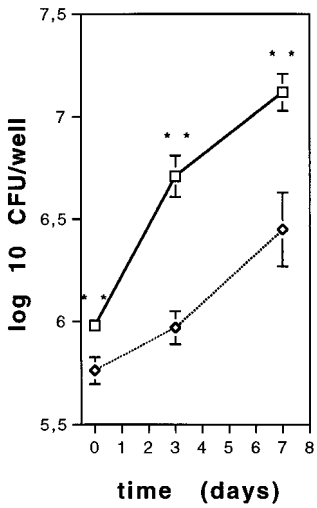

FIG. 1. Growth kinetics of *M. avium* ATCC 25291 (squares) and 1983 (diamonds) in RPMϕ cultured in vitro. The results shown are from a typical experiment. **, $P < 0.010$.

NK cell depletion and cytotoxicity assay. Peritoneal exudate cells were treated or not treated with the supernatants of GK 1.5 (anti-L3T4), 2.43 (anti-Lyt 2.2) and PK 136 (anti-NK cell) in the presence of complement, and NK cell activity was determined in an aliquot of each cell suspension by measuring the cytotoxic activity of the peritoneal cells against target YAC-1 cells, as described elsewhere (23), and quantitating the release of ^{51}Cr from the target cells after coincubation (4 h) at different target/effector cell ratios.

T-cell depletion and fluorescence-activated cell sorter (FACS) analysis. After treatment of peritoneal exudate cells with the supernatants of GK 1.5, 2.43, and PK 136 in the presence of complement, an aliquot of each cell suspension, treated and untreated, was stained with fluorescein isothiocyanate-conjugated rat anti-mouse CD4 or CD8 and/or phycoerythrin-conjugated hamster anti-mouse CD3-ε monoclonal antibody. After being stained, cells were analyzed in a FACScan apparatus (Becton-Dickinson). Among treated cells, the percentage of $CD4^+$ T cells was less than 0.5% and the percentage of $CD8^+$ T cells was less than 0.6%. The antibodies used for lysis were distinct from the ones used in the flow cytometric studies.

In vivo infection. Mice were intravenously infected with 10^6 CFU of *M. avium* ATCC 25291 or 1983. At 5 h after the inoculation and every 15 days throughout the experiment, anti-TNF antibodies were administered by intraperitoneal injection to a group of mice infected with each *M. avium* strain at a dose of 2 mg of antibody per mouse. At days 30 and 60 after infection, the spleen and liver of the mice were collected and the number of CFU was determined by serial dilution and by plating the tissue homogenates onto 7H10 medium (Difco).

Statistical analysis. The Student *t* test was used to compare pairs of data.

RESULTS

In vitro growth kinetics of *M. avium* ATCC 25291 and 1983 in mouse RPMϕ. The growth kinetics of the two *M. avium* strains in RPMϕ are shown in Fig. 1. RPMϕ monolayers were infected with either strain ATCC 25291 or strain 1983, and the growth of the bacteria inside the macrophages was studied for 7 days. Strain ATCC 25291 proliferated to a greater extent than did strain 1983 (5.39-fold at day 3, compared with 1.61-fold; 14.0-fold at day 7, compared with 5.13-fold), confirming our previous results on the characterization of the relative virulence of the two strains (28). Although the macrophage monolayers were infected with equal numbers of bacteria, strain ATCC 25291 was phagocytosed to a greater extent than was strain 1983.

Pattern of in vitro TNF production during the infection period. TNF activity present in the culture supernatants of uninfected or infected RPMϕ was determined at several time

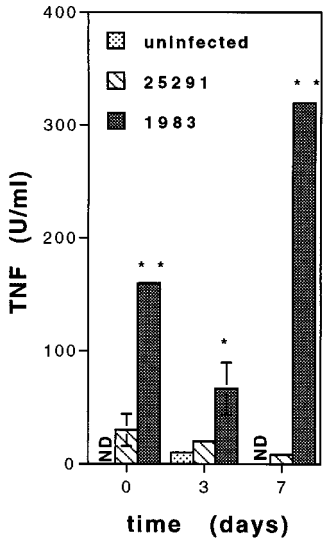

FIG. 2. TNF production by RPMϕ infected in vitro with *M. avium* ATCC 25291 or 1983 or left uninfected. TNF activity was measured in the supernatants by the L929 bioassay. ND, not detected (the assay sensitivity is 10 U/ml). **, $P < 0.010$; *, $P < 0.050$.

points (Fig. 2). Time zero activity was assessed immediately after the extracellular bacteria were washed out. Supernatants from *M. avium* 1983-infected RPMϕ showed higher TNF activity than did supernatants from *M. avium* ATCC 25291-infected or uninfected RPMϕ at all time points tested. Infection by strain 25291 led to a small but statistically significant increase in TNF secretion by RPMϕ up to 3 days postinfection as compared with untreated macrophages.

TNF production and *M. avium* growth in T-cell- and NK-cell-depleted RPMϕ cultures. To test the possible involvement of cytokines produced by T cells or NK cells that might be contaminating the macrophage cultures in the TNF production observed, we treated peritoneal cells, prior to adhesion, with anti-CD8, anti-CD4, and anti-NK cell antibodies in the presence of complement. The antibody treatment substantially decreased the numbers of $CD4^+$ and $CD8^+$ T cells present in the cell suspension as measured by FACS analysis (results not shown). NK-cell activity in the peritoneal cell suspension was undetectable either before or after the anti-NK-cell antibody treatment, as assayed by the YAC-1 cytotoxicity test (results not shown).

T-cell and NK-cell depletion of RPMϕ cultures did not change TNF production at day 7 (Fig. 3A), suggesting the existence of a direct stimulation of macrophages for TNF production by the bacteria. *M. avium* growth was also not altered by T-cell and NK-cell depletion (Fig. 3B).

TNF and anti-TNF treatment of RPMϕ infected in vitro with *M. avium*. When RPMϕ were treated with 5,000 neutralizing units of anti-TNF antibodies, strain 1983 growth increased to a level similar to that of strain ATCC 25291 but the growth of strain ATCC 25291 was unaffected (Fig. 4). Because *M. avium* ATCC 25291 induced low TNF production by RPMϕ, we treated ATCC 25291-infected RPMϕ with 500 U of TNF per day to test whether we could reduce the growth of this virulent strain by supplying the cytokine exogenously. We found that TNF treatment of ATCC 25291-infected RPMϕ did not interfere with bacillary growth (Fig. 5).

Effects of anti-TNF treatment in in vivo *M. avium* infection. *M. avium* ATCC 25291 proliferated progressively in the organs of untreated BALB/c mice; after a small amount of growth

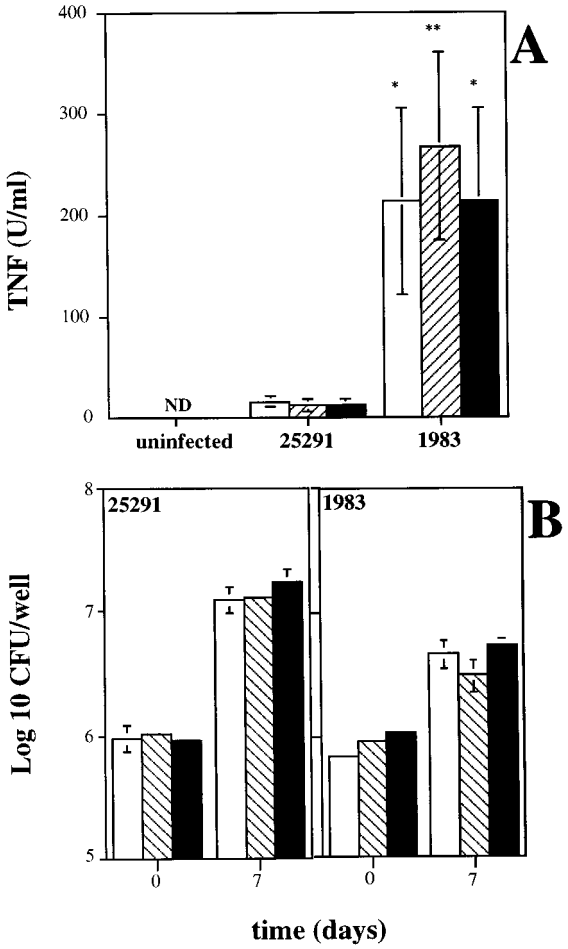

FIG. 3. Effects of T-cell depletion on in vitro TNF production by RPMϕ at day 7 postinoculation (A) and *M. avium* growth in in vitro-cultured RPMϕ (B). White columns, untreated adherent peritoneal exudate cells; hatched columns, peritoneal exudate cells treated with anti-CD4 and anti-CD8 antibodies before adhesion, in the presence of complement; black columns, peritoneal exudate cells treated only with complement before adhesion. The results shown are from a typical experiment; each column represents the mean of three wells. ND, not detectable. **, $P < 0.010$; *, $P < 0.050$.

observed until day 30 of infection in the liver and spleen, strain 1983 was slowly eliminated in the liver and its growth was arrested in the spleen. Both strains were slowly eliminated in the liver of untreated C.D2 mice, although strain 1983 was eliminated faster than strain ATCC 25291; strain ATCC 25291 was able to show a small amount of growth in the spleen of these mice, and strain 1983 was very slowly eliminated.

Neutralization of TNF in BALB/c mice infected with strain 1983 abrogated the clearance of the bacteria in the liver and caused a slight but significant increase in bacillary growth in the spleen at 30 and 60 days of infection. The same treatment in BALB/c mice infected with strain ATCC 25291 resulted in a minor, albeit significant, increase in mycobacterial growth only at 60 days of infection (Fig. 6). Such treatment in C.D2 mice had no effect on the growth of either strain 1983 or strain ATCC 25291 (Fig. 6).

DISCUSSION

The data presented here extended the results reported by Furney et al. (17) and our own results (3) on the involvement

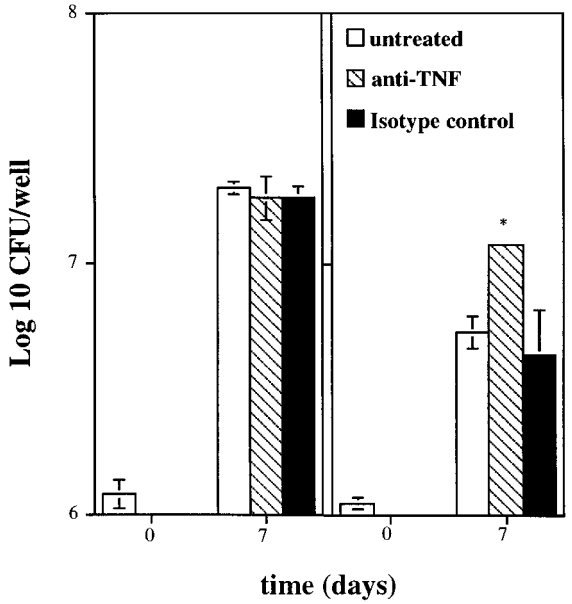

FIG. 4. Effect of anti-TNF treatment of in vitro-cultured RPMϕ on the growth of *M. avium* ATCC 25291 (left panel) and 1983 (right panel). At day 0 of infection, 5,000 neutralizing units of anti-TNF was added to each well. These results are from a typical experiment. *, $P < 0.050$ compared with the isotype control.

of TNF in the early control of *M. avium* infection by mouse macrophages. We found that the induction of TNF secretion by macrophages in vitro by *M. avium* was higher with a strain of low virulence than with one of high virulence. This is in agreement with a general trend found among different mycobacteria correlating virulence with lack of induction of TNF (11, 15, 17). These authors used different macrophage populations such as BMMϕ, casein-elicited peritoneal macrophages, and human macrophages. To focus on the relationships between the mycobacteria and the macrophages in the initial period of infection, we chose to study macrophages collected from the peritoneal cavity of untreated mice, since this macrophage population represents a resident macrophage population and is therefore the first population to make contact with the pathogen in vivo.

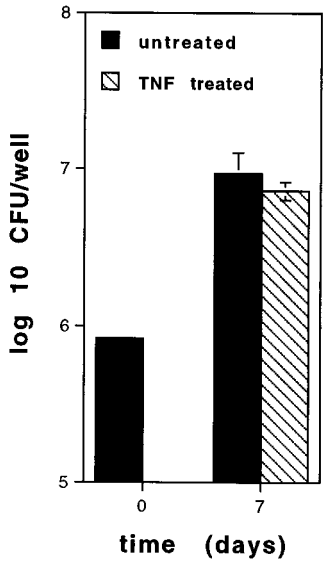

FIG. 5. Effect of TNF treatment of RPMϕ on the growth of *M. avium* ATCC 25291. TNF treatment consisted of daily addition of 500 U of recombinant murine TNF per well throughout the experiment. The results represent a typical experiment.

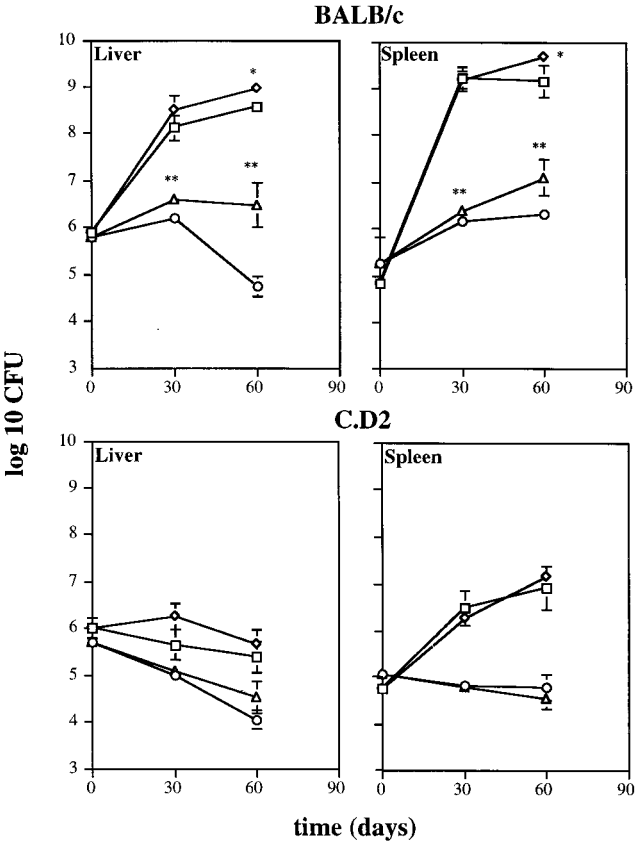

FIG. 6. Effect of anti-TNF treatment on the growth of *M. avium* ATCC 25291 or 1983 in vivo, in intravenously infected BALB/c (naturally susceptible) or C.D2 (naturally resistant) mice. Mice were given 2 mg of anti-TNF intraperitoneally at day 0 and every 2 weeks during infection. Squares, untreated mice infected with ATCC 25291; diamonds, anti-TNF-treated mice infected with ATCC 25291; circles, untreated mice infected with 1983; triangles, anti-TNF-treated mice infected with 1983. **, $P < 0.010$; *, $P < 0.050$.

In our system, TNF production by macrophages was most probably not dependent on stimulation by other cytokines, such as gamma interferon, produced by small numbers of contaminating T cells or NK cells since depletion of $CD4^+$, $CD8^+$, and $NK1.1^+$ cells did not affect TNF secretion. Although we cannot formally exclude that in vivo production of gamma interferon has not taken place in the peritoneal cavity of donor mice, it is rather unlikely that such a response had occurred in mice from our colony, which have never shown signs of natural infection. On the other hand, the results presented by Furney et al. (17) were obtained from BMMϕ, in which no contamination by nonmacrophage cell types has been reported, but nevertheless, TNF triggering by *M. avium* was proven.

The studies mentioned above (11, 15, 17) have not evaluated the actual role of the TNF produced after infection of the macrophages. Indeed, the hypothesis that avirulent strains were not able to grow because of the early production of TNF was based only on the observation of an association between virulence and lack of TNF secretion. We tested this hypothesis with neutralizing antibodies both in vitro and in vivo. Neutralization of TNF during infection of RPMϕ with strain 1983 in

vitro enhanced the growth of this low-virulence strain to a level similar to that of strain ATCC 25291 but had no effect on the growth of the latter strain. Thus, by inducing the synthesis of TNF by RPMϕ, strain 1983 was prevented from growing in the macrophages.

We also found that when TNF was exogenously added to the cultures, the growth of strain 25291 was not affected. This is in contrast to previous findings that showed that TNF alone induced bacteriostasis of *M. avium* ATCC 25291 in BMMϕ (2). We suggest that RPMϕ are less responsive to TNF than BMMϕ or that other cytokines might be involved in the induction of bacteriostasis. Conversely, strain ATCC 25291 could be inducing the unresponsiveness to TNF in RPMϕ. In this respect, it has been shown that a 33-kDa *M. avium* surface protein interferes with the regulation of transcription in macrophages, influencing the response of macrophages to stimulation with TNF (5, 21). Furthermore, interleukin-6 (6) was shown to down-regulate the expression of TNF receptors in the macrophage.

In vivo, TNF neutralization in BALB/c-mice infected with *M. avium* 1983 abrogated bacterial clearance in the liver and bacteriostasis in the spleen, suggesting the existence of a TNF-dependent antimycobacterial activity against strain 1983 in those organs. Neutralization of TNF in BALB/c mice infected with the highly virulent strain showed minor effects on its growth, which were observed later in infection when macrophage priming for TNF secretion is expected to have occurred (1). These data suggest that the early induction of macrophage TNF production by strain 1983 in vivo may be relevant in restraining the growth of this strain, when T-cell-dependent immunity is not yet established. However, TNF is probably not the only factor responsible for the low proliferation of strain 1983 in vivo, because this strain grew poorly compared with strain ATCC 25291 even in TNF-depleted mice.

The innate ability of C.D2 mice to restrict the growth of *M. avium* was independent of TNF, since the neutralization of this cytokine did not significantly affect the growth of both *M. avium* strains in these mice. This is in contrast to what has been postulated as the basis of the resistance to *Leishmania major* infection in *Bcg*^{r} (*Lsh*^{r}) animals reported by Blackwell et al. (8); in their model, BMMϕ from *Lsh*^{r} mouse strains released larger amounts of TNF in response to lipopolysaccharide and gamma interferon than did BMMϕ from *Lsh*^{s} mouse strains, correlating with higher leishmanicidal activity of *Lsh*^{r} macrophages. We suggest that the *Bcg* gene may essentially affect the antimycobacterial mechanisms of macrophages or their response to the cytokines rather than the autocrine regulation of the function of macrophages through cytokine release.

In conclusion, induction of TNF production by a low-virulence strain of *M. avium* (strain 1983) in resident macrophages seems to be relevant to the limiting of bacterial growth in vitro. In vivo, the capacity of strain 1983 to induce TNF production might be relevant for growth restriction of this strain, prior to the establishment of the T-cell response. On the other hand, a highly virulent strain (ATCC 25291) induces little, if any, TNF production in resident macrophages and TNF seems to play a role in growth restriction in vivo only after a T-cell response is established: either high doses of TNF are required and stimulate primed macrophages, or synergism with T-cell cytokines, like gamma interferon, is needed for TNF to be effective in limiting the growth of *M. avium* ATCC 25291. The relationship between avirulence and a TNF-dependent innate mechanism of resistance should not, however, be generalized to all isolates of *M. avium*. Susceptibility of different isolates of *M. avium* to the TNF-induced macrophage antimycobacterial mechanisms was previously shown to vary (2). Most importantly, other avirulent strains of *M. avium* have been shown to be eliminated by TNF-independent mechanisms (3). More extensive studies involving larger numbers of isolates are surely warranted.

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